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14. **ABSTRACT**

The 14-3-3 proteins are central to cell growth and have been implicated in the regulation of tumor development. While much is known about their mode of function in that they bind to ligands and affect their cellular activity, the regulation of 14-3-3s is not clearly understood. For this we have developed a cell-based assay to screen for modulators of 14-3-3 functions. Specifically we have screened a chemical compound library, and more recently as a follow up, a kinase ORF and a kinase siRNA library for molecules that affect the transport of 14-3-3 sigma from the nucleus to the cytoplasm. We have identified a number of candidate hits that we describe here and which we are currently in the process of characterizing.

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INTRODUCTION

Complex signaling networks play a critical role in normal and tumor cell growth and their regulation is often dependent on compartmentalization of signal transducing and effector proteins within such networks. For instance, proteins such as transcription factors are held in the cytoplasm until the cell receives an external signal at which time the protein moves rapidly into the nucleus. Conversely, the export of proteins from the nucleus can be similarly regulated to control the nuclear presence of a particular protein such as a DNA or RNA binding protein, chaperone or kinase. This regulated movement acts as a robust readout for cellular responses to signals and provides potential therapeutic targets.

Central to many signaling pathways are the small 14-3-3 adaptor molecules that bind as dimers to phophoserine and phosphothreonine in a multitude of ligands, including oncoproteins and tumor suppressors. Such binding can alter the ligands' activity and subcellular localization. Many 14-3-3 isoforms modulate the activity and localization of their binding partners by shuttling between the nucleus and cytoplasm [1]. As a result, 14-3-3 proteins regulate cellular processes such as apoptosis, cell cycle and mitogenic signaling and have been suggested to have anti-tumor properties.

14-3-3 functions

The 14-3-3 proteins constitute a family of abundant, widely expressed polypeptides. There are currently seven known isoforms in human cells. These bind to phosphoserine and phosphothreonine [2] [3]. The mechanisms of ligand regulation following 14-3-3 binding include impeding or facilitating protein-protein interaction (e.g. BAD and Bcl2; Raf and Bcr, respectively), altering catalytic activity (e.g. Tryptophan and Tyrosine hydroxylases), protecting from proteolysis (e.g. plant nitrate reductase) or from dephosphorylation (e.g. Raf and BAD). However, to date the most common mode of 14-3-3 function is the subcellular sequestration of binding targets. This regulatory mode has been observed in various proteins such as Kip1 [4, 5], Cdc25B [6, 7], Cdc25C [8, 9], Chk1 [10], BAD [11] and FOXO1 [12, 13]. It was initially thought that a putative NES within the 14-3-3 C-termini mediates the export of 14-3-3-ligand complexes [14]. However, recent findings question this "attachable NES model' and suggest that 14-3-3 proteins direct ligands out of the nucleus by exposing subcellular localization determinants within the bound cargo [12].

Regulation of 14-3-3

14-3-3 proteins are negatively regulated by phosphorylation. For instance, phosphorylation of 14-3-3ζ and 14-3-3σ inhibits dimer formation and impairs the ability of 14-3-3 to sequester ligands in the cytoplasm [15, 16]. Inhibition of 14-3-3 function by phosphorylation can probably be generalized to all isoforms, since the sites of phosphorylation (Ser58, Ser185, and Thr233; reviewed in [17]) are conserved.

To date, a number of kinases have been shown to phosphorylate 14-3-3. These include Calmodulin Kinase IIdC (CaM KIIdC) [16], Casein Kinase1 (CKI) [18], Sphingosine-Dependent Kinase 1(SDK1) [19], Akt [20], and Protein Kinase C's (PKC) [21, 22]. Recent reports suggest that activation of the Androgen Receptor (AR) and Epithelial Growth Factor Receptor (EFGR) result in 14-3-3σ phosphorylation and the accumulation of 14-3-3σ in the nucleus [23]. In these cases, 14-3-3 inactivation would seem to be part of an oncogenic signaling program. The outcome of phosphorylation as well as the effects of other upstream regulators, and the role they play in 14-3-3 activity remain poorly understood at a mechanistic level.

Role of 14-3-3 in cancer

The role of 14-3-3σ in cancer is paradoxical. The sigma isoform was identified as specifically absent in breast cancers [18], though apparently present in most other cancers. Huang et al. [23] found that 14-3-3σ was nuclear in prostate cancers and cytoplasmic in normal prostate tissue. In contrast, I find that14-3-3ς is primarily cytoplasmic in HeLa cells, and its role in export of FOXO1 from the HeLa cell nucleus would appear to be a pro-oncogenic activity. Also, 14-3-3σ sequesters Bax and blocks apoptosis in adriamycin-treated cells, which would appear to be a pro-oncogenic activity. The sigma isoform is also of interest because it is induced by p53 in response to DNA damage [18].

Lack of 14-3-3σ results in an inability to maintain the G2/M checkpoing after treatment with a DNA-damaging agent, and cell death through mitotic catastrophe [18].

14-3-3 and therapeutics

A correlation between 14-3-3 regulation and tumor growth control has been established, predominantly for the sigma isoform. For instance, p53 and BRCA1 both induce the expression of 14-3-3σ in response to DNA damage [24-26]. 14-3-3σ is necessary and its upregulation is sufficient to block cell cycle progression at the G2/M boundary upon DNA damage. Moreover, disruption of 14-3-3σ expression has been associated with immortalization of cells [27]. Interestingly, 14-3-3σ deficient cells have increased sensitivity to DNA-damage induced apoptosis [28].

The association of 14-3-3 inactivation with increased cell proliferation and sensitivity to apoptosis could be therapeutically exploited. For instance, a number of conventional anti-cancer drugs are selective for rapidly dividing cells and their efficiency is reduced in apoptosis-resistant cells [29]. Therefore, disruption of 14-3-3 activity by blocking its nuclear export or ligand binding will possibly enhance the effect of anti-cancer drugs. In this respect, inhibition of 14-3-3-ligand binding by short peptides increased cell sensitivity to apoptosis upon treatment with lower than conventional doses of cisplatin.

The emerging role of 14-3-3 in the regulation of cell response to DNA damage makes it an ideal target for cancer therapy. As a number of therapeutics function as DNA damaging agents, disruption of the G2/M checkpoint renders cells more vulnerable to cell death as this prevents cell cycle arrest to repair damaged DNA. In this respect, studies on the UCN-01 compound that inhibits the G2/M checkpoint by inhibiting Cdc25C phosphorylation on the 14-3-3 binding site are encouraging [30, 31]. Consequently, small molecules and cellular pathways that disrupt the activity of 14-3-3 will potentially exhibit synergistic effects when used in combination with DNA damaging regimens (e.g. chemotherapy and γ-irradiation).

I have previously proposed to study the regulation of 14-3-3 sigma nuclear export as a means to understand the regulation of 14-3-3 function and to identify small molecules that will be tested for therapeutic potential on a number of cancer cells including prostate cancer. In a previous screen for FOXO1a export inhibitors, our group had identified a series of inhibitory compounds. When tested one of these, TK10, shows an inhibitory effect on 14-3-3 sigma nuclear export. Interestingly, and contrary to the views at the time the proposal was written, TK10 inhibits 14-3-3 sigma in a manner independent of the general nuclear export CRM1 which was though to mediate 14-3-3 sigma export. Here I expand the screen to large scale compound libraries and show the identification of haloprogin as a new molecule that inhibits 14-3-3 sigma export. Additionally, two genomic screens of kinase libraries shed some light on potential regulators of 14-3-3 sigma function.

BODY

Determine the biological activity of the newly identified inhibitor of 14-3-3σ nuclear export TK10

I have previously shown that an inhibitor of FOXO1a nuclear export, TK10, inhibits the export of 14-3-3 from the nucleus while TK10 does not affect the general nuclear export through the CRM1 receptor, establishing that 14-3-3 sigma actually utilizes a CRM1-independent mechanism to exit the nucleus.
I have to date constructed a series of plasmids to study the effect of TK10 on 14-3-3 nuclear export. TK10 was shown to act through the PI3K/Akt pathway. For this, I am interested in studying whether expression of activated Akt interferes with the ability of TK10 to block 14-3-3 nuclear export. To this end, I have generated a myristylated Akt (myr-Akt). Upon expression of this construct, Akt is activated through its relocalization to the plasma membrane. The effect of this expression on 14-3-3 nuclear export in +/- TK10 cells will be studied in the future. Additionally I have constructed a FLAG-tagged version of the 14-3-3 ligand FOXO1a and showed that it is properly expressed in mammalian cells; FLAG-FOXO1a expressed from this vector localizes to the cytoplasm and upon treatment with LMB or TK10 the protein is trapped in the nucleus. The construct will be used to study specificity of other 14-3-3 sigma inhibitors identified in the screen described below.

Screen for small molecules that inhibit 14-3-3σ nuclear export at the ICCB

To elucidate the regulatory pathways that govern the nucleocytoplasmic transport of 14-3-3σ, I have developed a high-content high-throughput cell-based assay. The rationale being that 14-3-3σ is known to shuttle into and out of the nucleus during its life-time, the assay is set up to identify cells in which 14-3-3σ is trapped in the nucleus in response to drug treatment. The assay is performed in HeLa cells, which express high levels of the 14-3-3σ isoform that can be readily detected by immunofluorescence with a monoclonal antibody (CS112A) specific to the sigma isoform [12].

For the screen, cells were seeded in 384-well plates at 3,000 cells/well and allowed to adhere and grow for 24 hours, to reach 60-70% confluency. Under these conditions, 14-3-3σ localizes predominantly in the cytoplasm as detected by immunostaining. To screen for modulators of 14-3-3 localization, library compounds were added to cells by pin transfer and the plates incubated at 37 degrees for 1 hour. Following this incubation, the localization of 14-3-3σ was determined by immunostaining with the 14-3-3σ monoclonal antibody. Briefly, cells were fixed in 3.7% paraformaldehyde, permeabilized in 0.5% Triton X-100, and blocked with 3% BSA. This was followed by addition of 14-3-3σ specific antibodies.Visualization of the protein localization was achieved by adding Alexa Fluor 488-labelled anti-mouse antibodies. These steps were automated, allowing a many molecules to be screened with minimal effort. TK10, a previously identified FOXO1a export inhibitor that I found inhibits 14-3-3σ as well, was included in all subsequent screens as a positive control.

Images of multiple fields were obtained in an automated manner (Figure 1) and visually inspected to identify cells where 14-3-3σ was localized in the nucleus. Nuclear 14-3-3σ indicated a positive hit while cytoplasmic staining was scored as a negative result (Figure 1). All experiments were conducted in duplicate to minimize errors.

I screened approximately 30,000 small molecules from both commercial and public sources at the Harvard Institute of Chemistry and Cell Biology. From the original screen, I identified 7 compounds that reproducibly resulted in nuclear accumulation of 14-3-3σ. Upon initial characterization of these compounds, 4 are autofluorescent and have the ability to bind DNA, and therefore the signal I

detected in the nucleus was not specific to 14-3-3σ. These were dropped from further analysis. The remaining 3 were obtained from commercial sources (Figure 1) and one, haloprogin (Figure 2), has been studied in some detail as summarized below. The remaining compounds continue to be studied.

The nuclear export pathway for 14-3-3

The mechanism by which 14-3-3 is exported from the nucleus remains unclear. Early studies demonstrating 14-3-3 nuclear accumulation in response to treatment with leptomycin (LMB) indicated that 14-3-3 export was dependent on the major nuclear export receptor CRM1. However, the effects of LMB on 14-3-3 nuclear export were observed only at 100 nM and only after 18-24 hours [12]. I found that export of Rev-GFP, which use the CRM1 exporter, are normally inhibited at 1nM LMB and within 1 hour following treatment. Rev is an HIV protein with a canonical Nuclear Export Sequence and binds to CRM1 for export from the nucleus [32]; its export is thus LMB sensitive [13].

More recently, I and others showed that 14-3-3σ export is only marginally affected by LMB treatment in the same cells and under the same conditions as previously published suggesting an alternative export pathway [33, 34]. I also tested a number of other CRM1-blocking general export inhibitors identified in a screen for FOXO1 nuclear export inhibitors: none inhibited 14-3-3σ nuclear export. A different export receptor, Exportin 7, has been proposed as the primary nuclear export receptor for 14-3-3σ because 14-3-3σ binds Exportin 7 but not CRM1 in a Ran-GTP dependent manner [33]. I am currently exploring the hypothesis that haloprogin inhibits 14-3-3σ nuclear export by interfering with the Exportin 7 receptor.

Perform secondary assays to characterize the identified 14-3-3 export inhibitors

Haloprogin effect on the general CRM1-mediated nuclear export

I further studied the effects of haloprogin. This compound is a topical antifungal agent whose target is unknown. At concentrations as low as 20 μM, there was still an affect on 14-3-3 export in HeLa cells. I also determined the cell-type specificity of haloprogin action using the osteosarcoma cell line, U2-OS and the prostate cancer cell line 786-O. The levels of 14-3-3σ are similar to that of HeLa cells and can be readily detected with the CS112A antibody. Under conditions similar to those used for the original screen, haloprogin inhibited 14-3-3σ export from the nucleus in U2-OS and 786-O cells.

I found that haloprogin does not affect the export of other proteins that shuttle between the nucleus and the cytoplasm. In particular, this compound has no affect on the localization of Rev-GFP stably expressed in U2-OS cells. I also expressed a FLAG-tagged version of FOXO1 in HeLa cells, and found that LMB caused the accumulation of FOXO1 in the nucleus while haloprogin had no effect on FOXO1 localization.

Specificity of haloprogin towards the 14-3-3σ isoform

I studied the effect of haloprogin on other 14-3-3 isoforms. For this a number of isoform specific antibodies were obtained from commercial sources. Specifically antibodies for the gamma, tau, beta, and beta/zeta isoforms are available. I determined the optimal concentration for these antibodies in my assay. Through immunostaining studies, I determined that haloprogin exhibits an inhibitory effect on the export of the gamma and tau isoforms but not on the beta isoform. Interestingly haloprogin shows an inhibitory effect on the nuclear export of the isoforms detected by the beta/zeta antibodies, suggesting that the zeta isoform might be inhibited for nuclear export by haloprogin. This will be determined when reagents specific to this isoform become available or upon overexpression studies using epitope-tagged 14-3-3 zeta.

Studies aiming to identify kinases that play a role in the regulation of 14-3-3 nuclear export

I had previously suggested a high throughput multiplex kinase assay to identify kinases that might phosphorylate 14-3-3 and induce its nuclear trapping. Since then, other reagents have become available that can answer this question in a more quantitative high throughput manner. For this, I adapted the assay developed to a library of siRNA that target the predicted ~500 kinases in the human genome. The rationale being that a kinase siRNA that shows a similar phenotype as haloprogin on 14-3-3 might give us insight on the pathway or protein targeted by haloprogin as well as on the biology of 14-3-3 nuclear export regulation. This became more interesting with the emergence of recent findings that 14-3-3σ is trapped in the nucleus in response to signals that activate the EGFR and Androgen receptor pathways. Interestingly, when trapped in the nucleus 14-3-3σ is hyperphosphorylated. A number of studies have previously shown that 14-3-3 is phosphorylated by a series of known kinases, however there is no correlation to date between these phosphorylations and its activity or localization.

siRNA screening

To systematically screen the affects of many genes on 14-3-3 activity and localization, I am using a subset of siRNA libraries developed at the Novartis Institute for Biomedical Research (NIBR) to target the ~500 kinase expressing genes in the human genome. Two potential siRNA oligonucelotides have been designed and synthesized against a given gene. To increase the efficiency of silencing and screening, both siRNAs are pooled in one well in 384-well plates. The siRNAs from these libraries are transfected into HeLa cells at high efficiency. Through staining of fluorophore-coupled control siRNA transfected in control wells, I have determined that ~90% of the cells acquire the short oligonucleotides. Silencing through these siRNA proceeds for 48 hours before I immunostain to determine 14-3-3σ subcellular localization. I have developed a modified staining procedure to render this process high-throughput. Briefly, cells are fixed in 3.7% formaldehyde for 20 minutes followed by a permeabilization and a blocking step with Triton X-100/BSA. Cells are then incubated with 14-3-3σ specific antibodies followed by secondary fluorophore-coupled antibodies.

Fields of cells are then imaged on an ArrayScan microscope using a 10X lens. Images are then analyzed using an image analysis algorithm developed by Cellomics. Specifically the nuclear region is first determined by inspecting the signal from the DAPI channel. Subsequently, a ratio of cytoplasmic to nuclear staining in the FITC channel (14-3-3σ) is obtained. For each pool of siRNA, data from 500 cells is acquired from two duplicate plates to ensure statistical significance of the output. The compound haloprogin identified in the small molecule screen is used as a positive control.

The kinase set consists of two 384-well plates of siRNAs against ~500 kinases, both known and predicted from the human genome sequence. I have identified 16 siRNA targeted kinases that inhibit the nucleocytoplasmic transport of 14-3-3σ (Figure 3). For each of the hits identified, 8 siRNA targeting different regions of the hit gene are newly synthesized and re-assayed to determine specificity of the hit (Figure 8). Additionally, to eliminate off-target effects, results from the imaging screen are compared to results from the Q-PCR. Only siRNA hits that show significant knockdown of the target gene are further pursued. Also a visual inspection of the images is performed to double check the presence of the sought phenotype. To date, the preliminary data suggest that 2 kinases (MKNK2, a MAP kinase interacting serine/threonine kinase 2, and PDPK1, the 3-phosphoinositide dependent protein kinase 1) show on-target effect with knockdown activity that correlates with nuclear trapping of 14-3-3σ. These experiments are currently being validated.

Kinase ORF screen

In addition to the siRNA screen, I perform an overexpression screen using a library of kinase Open Reading Frames (ORF) to identify kinases that inhibit the nucleocytoplasmic transport of 14-3-3. For this screen I have obtained a collection of ~400 human kinase ORFs. These ORFs were mostly

PCR-amplified from the MGC collection [http://horfdg.dfci.harvard.edu] using sequence specific primers. Primers were designed to enable subcloning of PCR fragments in the Gateway system (Invitrogen) donor vectors through homologous recombination between the specific tails B1, B2 of the ORF and P1 and P2 sites of the Donor vector p201DNR to integrate the ORF in the vector and replace the ccdB toxic gene. Following the BP reaction, the Donor vectors are transformed into the DH5a bacterial strain and plasmid DNA is prepared. The constructs are then sequence-verified. The collection is plated in 96-well plates, making it compatible with our high-throughput screening method. Starting with these kinase ORFs/pDonor clones, I use homologous recombination between the tails L1 and L2 of the ORF/Donor vector and the R1 and R2 sites of a Destination vector to transfer the ORFs to a mammalian expression vector. Therefore, the ORFs were cloned in a Gateway altered Destination vector that encompasses a myc-tag in frame with the inserted ORF. The resulting products are constructs that express myc-fusion kinase ORFs from a CMV promoter in mammalian cells.

The myc-fusion constructs are transformed into the DH5alpha bacterial strain in a high-throughput manner and bacterial cultures containing the myc-ORF plasmids are grown in 96-well blocks in LB-Kanamycin for ~24 hours. From these cultures glycerol stocks are generated. The remainder of the cultures is processed using a QIAGEN Biorobot to obtain high quality plasmid DNA. The DNA is analyzed in a high-throughput manner using a 96-well compatible UV spectrophotometer and the yield and concentration of the DNA is determined.

Using the assays described above for siRNAs, I studied the effect of kinase overexpression on the subcellular localization of 14–3–3σ. Transient transfection of plasmid DNA into HeLa cells is performed using Fugene 6. I have optimized the transfection conditions for high-throughput screening in 96-well plates and determined that transfection of 50ng of plasmid DNA at a 1:6 ratio to Fugene 6 is most optimal. Under these conditions I observe high transfection efficiency after a 48-hour incubation. The low cytotoxicity of Fugene and the ability to use it in conjunction with serum eliminates a number of washing steps and is time-efficient in such high-throughput screens.

With the ORF transfected cells in hand, I assay for the localization of 14-3-3 by immunostaining procedures using the mouse monoclonal antibody CS112A as described previously. Briefly, cells will be fixed in 3% paraformaldehyde, permeabilized with 0.5% triton and blocked in 3% BSA. Then, anti-14-3-3 and anti-myc antibodies are added to the cells followed by appropriate secondary antibodies. Staining of the nuclei is achieved through the addition of DAPI. Scoring of hits is done by visual inspection of the plates as follows: the localization of 14–3–3σ is determined in cells that show positive myc-staining and the percent of transfected cells showing 14-3-3 nuclear staining is determined. ~ 100 cells or more are counted in each well to obtain statistically significant data.

To date I have screened ~ 200 kinases using the method described above and am currently in the process of cloning the remainder of the kinases into the Destination vector. Of these 200, I have identified five kinases (FGR, NAGK, LIMK1, LOC51231, and CHKL) that affect the localization of 14-3-3σ based on visual (Figure 4) and quantitative analysis in HeLa and U2-OS cells and a sixth (DCK) that appears to be specific for U2-OS cells.

Visually, I have observed that upon expression of these kinases 60% or more of the transfected cells manifest a nuclear 14-3-3σ phenotype as opposed to less than 5% of cells transfected with control DNA. I have complemented this study with quantitative analysis of the staining of 14-3-3σ in nucleus vs. cytoplasm and was able to recapitulate my data.

For a number of these kinases a link with 14-3-3 has been established. For instance, LIMK1 is a serine kinase that regulates actin filament polymerization through phosphorylation and inactivation of the actin-binding protein cofilin through binding to 14-3-3 [35, 36]. DCK is involved in the regulation of the supply of ribonucleotides for DNA replication and repair purposes: the role of 14-3-3σ in the DNA damage checkpoint has been exploited through its link to the CDC25 family of phosphatases [14, 37, 38]. These findings then validate the approach for finding new kinases involved in 14-3-3 regulation.

KEY RESEARCH ACCOMPLISHMENTS

Effect of TK10 on 14-3-3 export

- Constructed and expressed myr-Akt
- Constructed and expressed FLAG-FOXO1

Screen of compounds inhibitors of 14-3-3 sigma nuclear export

- Development of a cell-based assay to screen chemical compounds that inhibit 14-3-3 sigma export
- Adaptation of nuclear export inhibition assay to high-throughput screening
- Screening of 30,000 chemical compounds
- Identification of novel compounds that inhibit 14-3-3 sigma export
- Identification of false positive inhibitors

Secondary assays to characterize compounds

- Cell-type specificity of compounds
- Isoform-specificity of compounds
- Database search for haloprogin mode of action
- Effect of compounds on CRM1-mediated nuclear export

Genomic screen of siRNA kinase library for 14-3-3 sigma nuclear export inhibitors

- Optimization of library siRNA transfection
- Screening of 500 kinase siRNAs
- Identification of 16 kinase siRNA hits
- Q-PCR analysis of hits for off-target effect
- Identification of 2 kinases whose knock-down expression affect 14-3-3 sigma nuclear export

Genomic screen of an overexpression kinase ORF library for 14-3-3 sigma nuclear export inhibitors

- Generation of a kinase ORF library compatible with mammalian cell expression
- Optimization of high throughput DNA preparation
- Optimization of high throughput DNA transfection

- Screening of 200 overexpressed ORF kinases
- Identification of 6 kinases that inhibit 14-3-3 nuclear export

REPORTABLE OUTCOMES

Presentations

- Poster presentation at Systems Biology Retreat, Harvard Medical School
- Four Group Meeting presentation, Dr. Pamela Silver laboratory
- Presentation to the Functional Genomics Group at Novartis Institutes for Biomedical Research
- Presentation to the Vice-President of Research and Development at Ambion, Inc.

Funding applied for based on the work supported by this training grant

- NIH funding for Dr. Pamela Silver

Research opportunities applied for and/or received based on experience/training supported by this grant

- Opportunity to collaborate with Novartis Institutes for Biomedical Research to screen their kinase siRNA collection for inhibitors of 14-3-3 nuclear export
- Opportunity to collaborate with Ambion, Inc. Research and Development group to screen their microRNA collection for inhibitors of 14-3-3 nuclear export

CONCLUSIONS

I have set up a high throughput screening cell-based assay to understand the biology of 14-3-3 sigma regulation and the role these proteins play in controlling tumor development. The approach I have undertaken is based on first identifying small molecules inhibitors of 14-3-3 nuclear export. To this end I tested a series of small molecules that inhibit the export of FKHR, a 14-3-3 binding ligand, and identified TK10 as an inhibitor of 14-3-3 export. Interestingly, TK10 does not inhibit CRM1 export, suggesting that contrary to the current belief, 14-3-3 sigma is export in the nucleus, at least in part, by a mechanism that does not involve CRM1. I screened a library of 30,000 small molecules and identified 3 compounds that interfere with CRM1 nuclear export. Of these compounds, haloprogin is the most potent and therefore I chose to focus on its characterization in the initial phases of the project. To date I have shown that haloprogin inhibits 14-3-3 sigma nuclear export in a CRM1-independent manner, and that haloprogin is specific to some but not all 14-3-3 isoforms. I have determined the working conditions for the drug and tested its activity in multiple cell lines including prostate cancer derived cells. Because phosphorylation of 14-3-3 sigma seems to play a role in the translocation of the protein in and out of the nucleus, I initiated a number collaborations and performed an overexpression screen of a kinase library (work in progress) and an siRNA overexpression screen of all predicted kinases from the human genome. To date I have established a number of candidates whose overexpression or knock-down results in the inhibition of 14-3-3 sigma.

I am now moving into developing a number of secondary assays to further understand the mechanism of action of haloprogin. Additionally, I will study the effect of haloprogin and TK10 on the control of prostate cancer cell growth.

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SUPPLEMENTAL MATERIALS

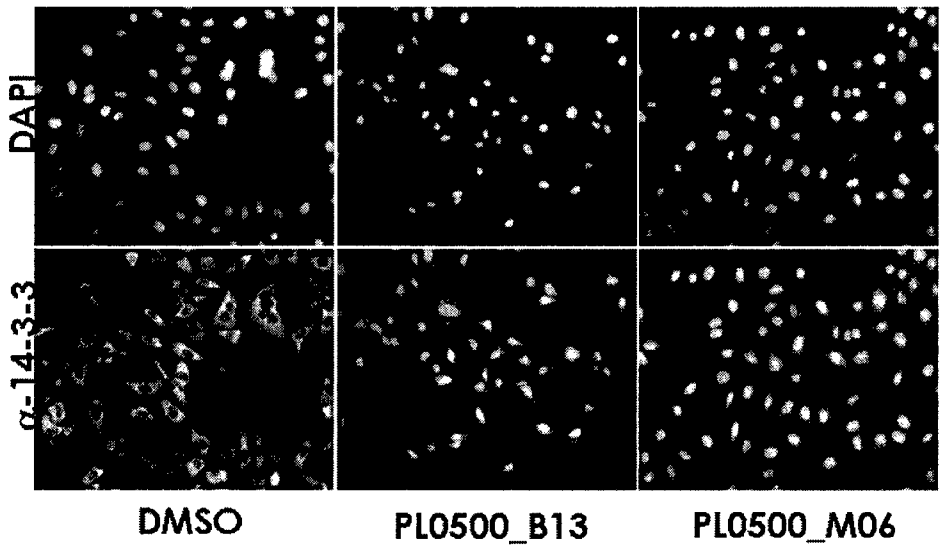

Figure 1: Representation of some of the hits from the initial chemical screen in HeLa cells. Top panel is DAPI staining. Bottom panel is 14-3-3 sigma staining following treatment with DMSO, hit B13 and hit M06. B13 and M06 show clearly 14-3-3 staining in the nucleus.

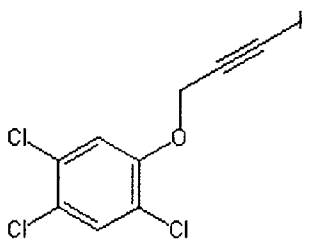

Figure 2: Structure of haloprogin, a small molecule drug approved for subcutaneous fungal treatment with unknown mode of action. Haloprogin inhibits the export of 14-3-3 from the nucleus of mammalian cells in a CRM1-independent manner.

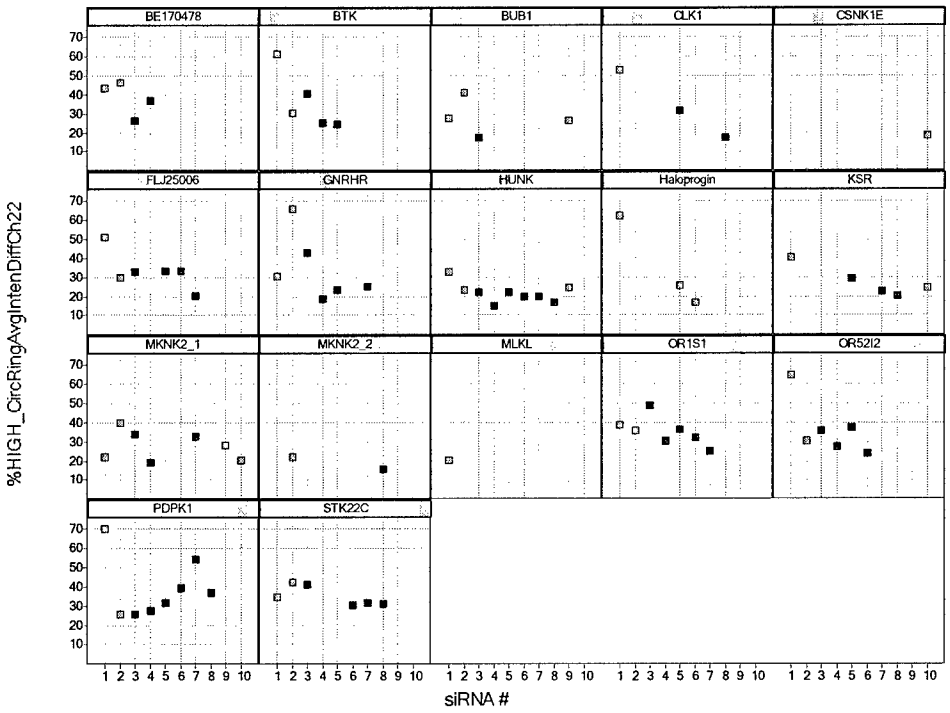

Figure 3: Effect of kinase siRNA expression on 14-3-3 sigma nuclear localization. For each of the 16 hits identified, 10 total oligonucleotides were retested (the original 2 from the screen, and 8 newly synthesized ones that target different regions of the gene). Results in this figure show the effect of each siRNA on 14-3-3 nuclear localization, in addition to the effect of haloprogin (row 2, chart 4 first column).

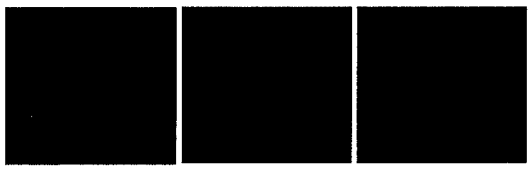

Figure 4: HeLa cells transfected with a representative kinase from the initial screen (LIMK1) and immunostained for 14-3-3 (green) and the myc-tagged kinase (red), and counterstained with DAPI (blue). Two of the four cells in the field were transfected, as evidenced by staining for the Myc tag on the kinase.